

MODULATION OF PHOSPHOFRUCTOKINASE ACTIVITY BY POSITIVE EFFECTORS

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1. Introduction

Phosphofructokinase is a major regulatory enzyme of glycolysis in many tissues. Its activity is controlled by a wide variety of effector molecules, including AMP, FDP, K^+ and NH_4^+ as activators, and MgATP and citrate as inhibitors (see [1] for a review).

MgATP is a substrate, and because of its additional inhibitory action, it has become accepted that it must bind to separate catalytic and inhibitory sites. The sigmoidal response of phosphofructokinase to fructose-6-phosphate (F6-P) is dependent upon the MgATP concentration, implying that occupation of the MgATP inhibitor site is necessary to demonstrate co-operative interactions between the F6-P binding sites. MgITP will also serve as a phosphate donor, but is non-inhibitory even at high concentrations [2]. Additionally, the sigmoidal response to F6-P is abolished when MgITP is substituted for MgATP. It seems probable that MgITP does not bind to the ATP inhibitor site [3].

The role of AMP as an activator of phosphofructokinase has been well established [4–6]. Recently, evidence has been presented suggesting that NH_4^+ may also be of considerable physiological importance as an activator [7, 8]. In this paper, by utilizing both MgATP and MgITP as substrates, we present evidence showing that AMP, NH_4^+ and K^+ interact with separate binding sites, and thereby modulate phosphofructokinase activity in a manner that may be of physiological importance.

2. Materials and methods

All substrates, nucleotides and coupling enzymes were from Boehringer Mannheim, G.F.R. NH_4^+ and K^+ were the chloride salts and were of analytical grade.

Phosphofructokinase was assayed in 50 mM Hepes–NaOH buffer pH 7.0, containing ATP, F6-P and effectors as indicated. Mg^{2+} was always 3 mM in excess over the ATP concentration [9]. Phosphofructokinase activity was measured by the decrease in extinction of NADH at A_{340} , using aldolase, triose-phosphate isomerase, and α -glycerophosphate dehydrogenase, approximately ten times in excess of the observed activity of phosphofructokinase. Coupling enzymes were dialyzed extensively against 50 mM Tris–Cl pH 8.0 containing 0.05 mM EDTA, to remove $(NH_4)_2 \cdot SO_4$.

Pig spleen phosphofructokinase was purified as far as the 'wash' stage of the method of Massey and Deal [10], to a specific activity of 4–8 i.u./mg protein (assayed in 50 mM Hepes–NaOH, pH 8.0, containing 0.4 mM ATP, 2.0 mM F6-P and 3.0 mM Mg^{2+}). The partially purified enzyme was dialyzed against 80 mM potassium phosphate pH 8.0, containing 1 mM EDTA. It was stable in this buffer at 4°C for 2–3 months; after one month only 10–15% of the initial activity had been lost. The enzyme was further purified by DEAE-Cellulose chromatography to a specific activity of at least 15 i.u./mg protein. All the results here were obtained with the 'washed' enzyme. Full details of the modified purification procedure will be given elsewhere.

Table 1
Synergistic activation of phosphofructokinase by NH_4^+ and AMP.

NH_4^+ (0.175 mM)	NH_4^+ (1.75 mM)	AMP (1.0 mM)	PFK activity		In MgITP (U/ml)	(% Stim.)
			In MgATP (U/ml)	(% Stim.)		
—	—	—	0.256	0	0.246	0
+	—	—	0.569	120	0.610	150
—	+	—	1.003	290	0.819	230
—	—	+	0.453	90	0.273	10
+	—	+	0.919	260	0.567	120
—	+	+	1.367	430	0.831	240

The stock phosphofructokinase solution was diluted in 80 mM potassium phosphate buffer. Assays were conducted as in the Materials and methods section, but with 0.2 mM F6-P, 1.1 mM ATP or ITP, and 4.0 mM Mg^{2+} .

3. Results

In table 1 the ability of AMP and NH_4^+ to synergistically activate phosphofructokinase is examined (pig spleen phosphofructokinase has an apparent K_A for NH_4^+ of 0.35 mM and an apparent K_A for AMP of 0.028 mM: P. Hickman, unpublished results). The two concentrations of NH_4^+ used in this experiment are therefore non-saturating and near-saturating respectively. The AMP concentration is saturating. Clearly NH_4^+ will stimulate when either MgATP or MgITP is the substrate, whilst AMP will stimulate only in the presence of MgATP. Thus there is a fundamental difference in the mode of activation by the two effectors: activation by AMP is dependent on the occupation of an ATP inhibitor site by inhibitor, whereas NH_4^+ activates independently of the occupation of this site. It is also clear that NH_4^+ and AMP act synergistically when each ligand is present at saturating concentration.

Table 2 examines the synergism between NH_4^+ and K^+ under the same experimental conditions. Clearly both NH_4^+ and K^+ stimulate phosphofructokinase activity when MgITP is used as the phosphate donor, and the activation by one ligand can be demonstrated in the presence of saturating concentrations of the other. (Pig spleen phosphofructokinase has an apparent K_A for K^+ of 11 mM: P. Hickman, unpublished results). This phenomenon has already been noted [7]. It is uncertain whether the reduced synergism in the presence of MgITP is of significance, as this particular measurement has only been made once.

The above results demonstrate that the binding sites for the three effectors are probably separate. To examine the effect of these ligands on the co-operative behaviour of phosphofructokinase, Hill coefficients [11] were determined in the presence and absence of the effectors. When phosphofructokinase activity was titrated with F6-P, the Hill coefficient changed only

Table 2
Synergistic activation of phosphofructokinase by NH_4^+ and K^+ .

NH_4^+ (10 mM)	K^+ (100 mM)	PFK activity		In MgITP (U/ml)	(% Stim.)
		In MgATP (U/ml)	(% Stim.)		
—	—	0.31	0	0.26	0
+	—	1.47	374	1.61	619
—	+	2.37	663	2.26	871
+	+	3.61	1161	2.58	994

Preparation of the enzyme, and assays were conducted as in table 1.

slightly, from 1.90 to 1.78 in the presence of K^+ . A similar result was found with NH_4^+ . When, however, the same titration was done in the presence of AMP, the Hill coefficient fell to 1.12. This result is taken to mean that AMP reduced the $MgATP$ -mediated co-operative interaction between F6-P substrate sites.

When phosphofructokinase is highly diluted, at acid pH, and in the presence of high concentrations of $MgATP$, it is rapidly inactivated [12]. In an experiment conducted at pH 6.70, in the presence of 3 mM $MgATP$ and 80 mM K^+ , AMP was far more effective than NH_4^+ in slowing down this inactivation process. In the presence of 0.5 mM AMP, the half-time of inactivation was found to be 116 sec, whereas in the presence of 2.0 mM NH_4^+ it was only 48 sec.

4. Discussion

The results presented above are consistent with the idea that AMP, NH_4^+ and K^+ have independent binding sites on phosphofructokinase. More importantly, they demonstrate that AMP acts in competition with $MgATP$ at the ATP inhibitor site, whilst the other effectors act by binding at independent sites, presumably by inducing a conformational change in the enzyme structure, or by altering the equilibrium position of pre-existing active and inactive forms.

With pyruvate kinase, NH_4^+ and K^+ bind to the same site with the same affinity [13], and it was felt that this might also be the case with phosphofructokinase. However, the very different activation affinities, the results presented above, and the results of Abrahams and Younathan [7], show that this expectation is not justified. The K^+ levels in most mammalian cells are near the saturation level for phosphofructokinase, so for NH_4^+ to exert significant additional activation, it must have its own independent binding site.

Our finding that AMP is more effective than NH_4^+ in delaying inactivation of phosphofructokinase, is of interest in light of the proposed association/dissociation mechanism for regulating phosphofructokinase activity [9,12,14,15]. The results presented in this

paper suggest that there are two separate mechanisms for regulating phosphofructokinase activity. Firstly, an association/dissociation mechanism, mediated primarily by $MgATP$ binding to the ATP inhibitor site and, secondly, 'fine' modulation of the activity of the associated form of the enzyme by the binding of specific ligands at additional allosteric sites. The role of inhibitors has not been considered here, but clearly they too could act in the same general manner.

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